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MINIREVIEW

Inactivation Strategies for *Clostridium perfringens* Spores and Vegetative Cells

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ABSTRACT *Clostridium perfringens* is an important pathogen to human and animals and causes a wide array of diseases, including histotoxic and gastrointestinal illnesses. *C. perfringens* spores are crucial in terms of the pathogenicity of this bacterium because they can survive in a dormant state in the environment and return to being live bacteria when they come in contact with nutrients in food or the human body. Although the strategies to inactivate *C. perfringens* vegetative cells are effective, the inactivation of *C. perfringens* spores is still a great challenge. A number of studies have been conducted in the past decade or so toward developing efficient inactivation strategies for *C. perfringens* spores and vegetative cells, which include physical approaches and the use of chemical preservatives and naturally derived antimicrobial agents. In this review, different inactivation strategies applied to control *C. perfringens* cells and spores are summarized, and the potential limitations and challenges of these strategies are discussed.

KEYWORDS *Clostridium perfringens*, spores, vegetative cells, inactivation, antimicrobial agents, food poisoning

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Clostridium perfringens is an anaerobic, spore-forming bacterium and can be found ubiquitously in the environment, including the guts of humans and other animals (1, 2). Although most strains of this species do not cause any harm to human and animals, a few of them are of concern due to their ability to cause a variety of histotoxic and gastrointestinal (GI) diseases (3, 4). *C. perfringens* can produce as many as 17 different toxins, but there is not a single strain that produces all of these toxins (3). Depending on the production of four major toxins (alpha, beta, epsilon, and iota), *C. perfringens* strains are classified into five toxin types (types A to E) (3). About 5% of global *C. perfringens* type A isolates produce *C. perfringens* enterotoxin (CPE), which is the major virulence factor for the pathogenesis of *C. perfringens*-associated food poisoning (FP) and nonfoodborne (NFB) GI diseases (3–6). Interestingly, most of the reported FP cases have been associated with *C. perfringens* isolates harboring the CPE-encoding gene (*cpe*) on the chromosome (C-*cpe* isolates), whereas isolates that possess *cpe* on the plasmid (P-*cpe* isolates) are linked to the occurrence of NFB GI diseases such as antibiotic-associated diarrhea and sporadic diarrhea, with some exceptions (7–9).

In addition to producing CPE, *C. perfringens* FP isolates have the ability to form spores that are highly resistant to various stress factors such as high temperature, high pressure, toxic chemicals, and radiation (10). However, *C. perfringens* vegetative cells are less resistant to these stress factors than their spore counterparts, and these resistance phenotypes vary among strains and growth conditions (11–13). For example, vegetative cells and spores of *C. perfringens* type A C-*cpe* isolates exhibited significantly higher

heat resistance than those of P-*cpe* and *cpe*-negative isolates (11, 12, 14). Besides resistance to heat, vegetative cells and spores of C-*cpe* isolates possess higher resistance to various food preservative measures, including low temperature, osmosis-induced stress, and nitrite, than those of the P-*cpe* isolates (13, 15). These facts suggest the importance of developing an effective strategy that could kill or inhibit the growth of different strains of *C. perfringens* simultaneously.

The possession of high resistance properties facilitates the survival of *C. perfringens* spores in food vehicles, where they undergo germination and outgrowth to revert to vegetative cells and then cause FP illness in humans upon consumption of these contaminated foods (2). *C. perfringens* is currently ranked as the third most common foodborne pathogen in the United States (16), and it is estimated that almost a million cases of food-associated illnesses occurred annually in the United States, implicating *C. perfringens* as an etiological agent (17). This high incidence would subsequently lead to substantial losses in terms of economics, manpower, and medical expenses (18). Therefore, to minimize these substantial economical losses, strategies are being developed to inactivate the spores and vegetative cells of *C. perfringens*, especially in food industries and food products. In this review, we discuss the current status of different approaches to effectively inactive the vegetative cells and spores of *C. perfringens*.

PHYSICAL APPROACHES

C. perfringens vegetative cells can easily be killed by introducing various abusive physical conditions, but their spore counterparts are difficult to inactivate with these treatments. However, studies have shown that either the manipulation of physical conditions or combined treatment with two or more stress factors was able to inactivate the spores (15, 19–21). Here, we discuss several of the physical parameters that can be used to inactivate spores and vegetative cells and their prospects for use in the food industry.

Thermal treatment. Thermal treatment is one of the most common ways of sterilizing the products, as excessive heat destroys the majority of the bacterial cells. It has been reported that *C. perfringens* spores are highly heat resistant (22), although the resistance patterns vary considerably with the strain and growth conditions, such as medium and incubation temperature (11). However, a significant inactivation of *C. perfringens* spores can be achieved by exposing them to high temperature for a longer period of time (23, 24). Wang et al. showed that more than 90% of *C. perfringens* spores were inactivated when they were incubated in water at 90 to 100°C for 10 to 30 min (23). Another report demonstrated that higher temperatures (110°C) were required to reduce the number of *C. perfringens* spores in the meat system (24) (Table 1). The introduction of other treatments (Table 1) in combination with moderate and high temperatures was also effective in the inactivation of both vegetative cells and spores of *C. perfringens* (19–21). For example, (i) implementation of ozone treatment followed by heat effectively inactivated both vegetative cells and spores of *C. perfringens* in a meat product (19), (ii) the simultaneous use of both thermal treatment and ultrasound or thermal treatment followed by ultrasound significantly increased the effectiveness of thermal inactivation of *C. perfringens* spores in a beef slurry (20), and (iii) pretreatment with gamma radiation followed by thermal treatment significantly decreased the number of spore counts (but interestingly, the reverse was not effective) (21).

Pressure treatment. High hydrostatic pressure (HHP) is one of the most widely used pasteurization techniques for food products other than temperature-dependent pasteurization. HHP treatment of food is more effective in killing of vegetative cells, and to some extent bacterial spores, than the conventional thermal processing of foods. It also retains the nutritional and sensory quality of the food products and consumes less energy than the other processing techniques (25). A combined treatment with HHP (650 MPa), temperature (75°C), and low pH (4.75) resulted in a 5.1-fold reduction of spores of *C. perfringens* type A P-*cpe* isolates but was not as effective (2.8-fold reduction) against spores of *C. perfringens* C-*cpe* isolates in laboratory medium (15). However, Gao et al. showed that the combined effects of HHP (654 MPa), heat (74°C), and nisin

TABLE 1 Effects of different inactivation strategies on the inhibition of *C. perfringens* cells and spores

Inactivation strategy	Matrix[a]	Treatment	Inhibitory effect	Reference(s)
Physical approaches				
Temp	Pork luncheon roll	1.3 min at 70°C for vegetative cells and 36 s at 110°C for spores	6-log reduction of spores and vegetative cells[b]	24
Temp and ozone	Beef surface	5 ppm aqueous ozone at 75°C	1–2-log CFU/g reduction of spores and vegetative cells	19
Temp and ultrasound	Beef slurry	Thermosonication (24 kHz, 0.33 W/g) at 75°C for 60 min	≤1.5-log reduction of spores	20
Temp and radiation	Buffer	Gamma radiation (0.7 Mrad) followed by thermal treatment at 103°C	6-log reduction of spores	21
HHP and temp	Poultry meat	586 MPa, 73°C for 10 min after spore germination with ≥50 mM L-asparagine and KCl mixtures	2–4-log CFU/g reduction of spore germination and outgrowth	103
HHP, pH, and temp	Buffer	650 MPa, 75°C at pH 4.75	1–5-fold reduction of spores for C-*cpe* isolates, 4–6-fold reduction for P-*cpe* isolates	15
Chemical agents				
Nitrate and nitrite	Broth	≥0.3% (wt/vol) $NaNO_2$	1–4-log CFU/ml reduction in vegetative cells and spores	13
	Broth	100 ppm $NaNO_2$ at 11°C	4-log CFU/ml reduction of spores and vegetative cells	30
	Ham	200 ppm $NaNO_2$	2–4-log CFU/g reduction of spores and vegetative cells[b]	29
Sorbic acid	Buffer	1% (vol/vol) sodium sorbate	30–70% inhibition of spore germination for C-*cpe* isolates, and 50–90% inhibition for P-*cpe* isolates	39
Benzoic acid	Buffer	1% (vol/vol) sodium benzoate	30–70% inhibition of spore germination for C-*cpe* isolates, and 98% inhibition for P-*cpe* isolates	39
Lactic acid	Tajik-sambusa	2% (wt/wt) potassium lactate	1–2-log CFU/g reduction of spore germination and outgrowth[b]	44
	Injected pork	≥2% (wt/wt) calcium lactate, ≥3% (wt/wt) potassium lactate, or ≥3% (wt/wt) sodium lactate	4–6-log CFU/g reduction of spore germination and outgrowth[b]	45
	Injected turkey	≥2% (wt/wt) calcium lactate, ≥3% (wt/wt) potassium lactate, or ≥3% (wt/wt) sodium lactate	4–6-log CFU/g reduction of spore germination and outgrowth[b]	45
	Uncured ground turkey	2% (wt/wt) potassium lactate	2–4-log CFU/g reduction of spore germination and outgrowth[b]	42
	Sous-vide chicken	≥1.5% (wt/wt) sodium lactate	3–6-log CFU/g reduction of spore germination and outgrowth[b]	43
Acetic acid	Roast beef	≥2% MOstatin LV1	6–7-log CFU/g reduction of spore germination and outgrowth[b]	48
	Ground turkey roast	2.5% MOstatin V or 3.5% MOstatin LV	3–5-log CFU/g reduction of spore germination and outgrowth[b]	49
Phosphates	Poultry meat	1% (wt/vol) sodium tripolyphosphate	2–3-log CFU/g reduction of spore germination and outgrowth[b]	52

(Continued on next page)

TABLE 1 (Continued)

Inactivation strategy	Matrix[a]	Treatment	Inhibitory effect	Reference(s)
Natural antimicrobials				
Plant				
Cinnamaldehyde	Cooked ground beef and turkey	1% (wt/wt) cinnamaldehyde	3–4-log CFU/g reduction of spore germination and outgrowth[b]	61, 62
Carvacrol, thymol, and oregano oil	Cooked ground beef and turkey	2% (wt/wt) carvacrol, 2% (wt/wt) thymol, and 2% (wt/wt) oregano oil	3–5-log CFU/g reduction of spore germination and outgrowth[b]	61, 62
Tannins	Broth	≥5 mg/ml chestnut tannins	Up to 7-log CFU/ml reduction of vegetative growth	72
Green tea	Cooked ground chicken and pork	2% (wt/wt) green tea extracts	3–4-log CFU/g reduction of spore germination and outgrowth[b]	76
Animal				
Fatty acids	Buffer	1 mg/ml lauric acid	>7-log reduction of vegetative growth	79
Lysozyme	Broth	156 μg/ml lysozyme from hen egg white	Complete inhibition of vegetative cells	85
Chitosan	Cooked ground beef and turkey	3% (wt/wt) chitosan	4–5-log CFU/g reduction of spore germination and outgrowth[b]	90
Microbial				
Nisin	Broth	≥10 μM nisin	Complete inhibition of spore outgrowth and vegetative growth	92
Lacticin	Fresh pork sausage	2,500 AU/g lacticin 3147 with 2% (wt/vol) sodium lactate and/or 2% (wt/vol) sodium citrate	3–4-log CFU/g reduction of spore germination and outgrowth[b]	95

[a]Different growth media were used for the inactivation strategies. Most of the experiments were performed with different meat products, as *C. perfringens* is a common pathogen in food products, especially in meats.
[b]The reduction value indicates the number of spores and/or cells compared to the total number of spores and/or cells of control samples (with no treatment).

(328 IU/ml) could effectively enhance the inactivation activity of HHP toward *C. perfringens* spores in milk products (26).

CHEMICAL AGENTS

Different types of chemical agents have been proven to be effective in controlling *C. perfringens*. Most of the studies have been focused on chemicals that can be used in food items as preservatives as well as flavor and color enhancers (27). Here, we discuss several of the common chemicals that have been used as food additives as well as food preservatives and have implications for the inhibition of *C. perfringens* spores and vegetative cells (Table 1).

Nitrate and nitrite. Nitrate and nitrite are well-known chemical preservatives for food to control spore-forming bacteria and have been used mostly in meat, fish, and cheese products for long periods of time (1, 27). These are also known as food additives, especially in cured meat, where nitrate and nitrite provide flavor and color stabilization (27). Nitrite can exert its antimicrobial effect against both vegetative cells and their spore counterparts, although spores are generally much more resistant to nitrite than growing cells (28). Nitrite affects the germination and outgrowth of *C. perfringens* spores in different types of meat products, and it has been reported that conventionally cured meat products, which have nitrite supplements, showed inhibition of the growth of *C. perfringens* (29, 30). However, commercially available organic meat products have the potential to support growth of *C. perfringens* (31, 32). Although nitrite is a very good antimicrobial agent, unfortunately its use in food products is limited due to its ability to form carcinogenic derivatives (33). This led to a search for alternative nitrite sources from natural compounds, although these compounds are not as effective as those used in conventionally cured meat for inhibition of the outgrowth of *C. perfringens* spores (34).

Organic acids. Organic acids are used as food preservatives and have been found to act as inhibitory agents for *C. perfringens* growth. Two of the most common and oldest organic acids used in food industries are sorbic acid (sorbate) and benzoic acid (benzoate). Both of these organic acids and their salts have been listed as generally recognized as safe (GRAS) compounds as food preservatives, with permissible concentrations approved by the U.S. Food and Drug Administration (FDA) (http://www.accessdata.fda.gov/scripts/fdcc/?set=SCOGS). Benzoates are one of the most widely used chemical antimicrobials because of their low cost, easy incorporation into foods, lack of impartment of color upon addition, and low toxicity (35, 36). However, sorbates are known mainly as antifungal agents. Tompkin et al. reported no difference in *C. perfringens* viability in presence or absence of potassium sorbate (37), but a follow-up study reported successful inhibition of *C. perfringens* growth by potassium sorbate in cooked sausage (38). Recently, our group showed the inhibitory effects of sorbate and benzoate derivatives against *C. perfringens* spore germination, outgrowth, and vegetative growth in both rich medium and a meat model system (39). Although the permissible levels of sorbate (0.3%, vol/vol) and benzoate (0.1%, vol/vol) could arrest *C. perfringens* spore outgrowth, higher concentrations (1%, vol/vol) are needed for the inhibition of spore germination in rich medium (Table 1) (39). Nevertheless, the permissible concentrations of sodium benzoate and potassium sorbate failed to control *C. perfringens* spore germination and outgrowth in cooked poultry meat during improper storage at 37°C, and this might result from the weak dissociation of the antimicrobials, rendering them ineffective against target microorganisms (39, 40).

Lactic acid and its derivatives of different salts have been reported to inhibit the germination and outgrowth of *C. perfringens* spores in different meat products, including injected turkey, injected pork, *sous-vide* chicken products, and tajik sambusa, under various abusive conditions (41–45). The lactate group represents a group of primary compounds responsible for the inhibition of germination and outgrowth of spores of *Clostridium* spp. (41) and could be utilized as an alternative to nitrite to warrant product safety during extended cooling of uncured meats (42). The addition of a 1.5% or higher concentrations of sodium lactate in marinated *sous-vide*-cooked chicken breast products led to the delay in the germination and outgrowth of spores of enterotoxigenic *C. perfringens* during storage at 19 and 25°C (43). Potassium lactate at 2% effectively restricted growth of a spore cocktail from 3 different *C. perfringens* strains during extended cooling of cooked uncured ground turkey breasts (42). However, calcium lactate was more effective than sodium lactate and potassium lactate in controlling germination and outgrowth a of spore cocktail from 3 *C. perfringens* strains in injected pork during a deviated chilling regimen (41).

Acetic acid, which is mostly used as vinegar, has been successfully applied for microbial growth inhibition and cell viability reduction for both Gram-positive and Gram-negative bacteria in different types of foods (46). Vinegar has been used as a condiment and food ingredient for the purpose of flavoring and preserving foods for thousands of years (47). A typical vinegar contains 5 to 40% acetic acid and other compounds that give the characteristic aroma (46). Recently, it was reported that a blend of vinegar and buffered lemon juice concentrate (MOStatin LV1) at different concentrations (0%, 2%, and 2.5%) was highly effective in controlling growth of *C. perfringens* spores in reduced-NaCl roast beef during abusive exponential cooling, regardless of the level of NaCl added and the cooling time used (48). The buffered vinegar (MOstatin V) at 2.5% and a blend of buffered lemon juice concentrate and vinegar (MOStatin LV) at 3.5% could also inhibit *C. perfringens* spore germination and outgrowth in ground turkey roast containing minimal ingredients (49).

Phosphates. Long-chain inorganic phosphates (polyphosphates [polyPs]) and the orthophosphate derivative blends have been extensively used in meat and dairy products for many years (50). These phosphates and their derivatives are used as food additives owing to their functional aspects regarding emulsification, stabilization, oxidation prevention, and flavor protection and, most importantly, as antimicrobial

TABLE 2 Antimicrobial activities of selected essential oils against *C. perfringens*

EO	MIC (μg/ml)	Reference
Cinnamaldehyde	140	60
trans-Cinnamaldehyde[a]	167	65
Thymol	375	63
Carvacrol	375	63
2-*tert*-Butyl-6-methyphenol[a]	175	65
Chamomile roman oil[a]	450	65
Citral oil[a]	275	65
Citronellal[a]	400	65
Geraniol[a]	450	65

[a]The MIC_{95} is shown.

agents. Antimicrobial activities of variable-length phosphates (ortho-, pyro-, and polyphosphates) have been shown against many different bacteria, including *C. perfringens* (51–53). Our group evaluated the inhibitory effects of various polyPs on *C. perfringens* and demonstrated the following: (i) polyP significantly inhibited sporulation of *C. perfringens* by reducing sporulating cells by ~5 to 6 $\log_{10}$; (ii) while *C. perfringens* spores were able to germinate in the presence of 1% sodium tripolyphosphate (STPP), their outgrowth was significantly inhibited; and (iii) STPP (at levels of ≥0.8%) exhibited antimicrobial activity against a spore cocktail from five different *C. perfringens* C-*cpe* isolates in chicken meat (52) (Table 1). Singh et al. found that the combination of sodium acid pyrophosphates from different sources were more effective than tetrasodium pyrophosphate in inhibition of *C. perfringens* growth in different meat products during abusive chilling conditions (53). However, the efficacy of these pyrophosphates depends on the type of meat product and dose of pyrophosphate (53).

NATURAL ANTIMICROBIALS

There are antimicrobial agents for inhibiting *C. perfringens* growth that are derived from natural sources, including animals, plants, and microorganisms (54, 55). Numerous researchers have studied the naturally occurring antimicrobial agents with regard to their properties of inhibition and inactivation of spoilage and disease-causing microorganisms (54, 55). The results revealed that many of them demonstrate a strong bactericidal effect and some of them are also inhibitory against spores of food-related bacteria; thus, there is a promising potential of these natural antimicrobial agents for being used as food preservatives. Table 1 listed some of the natural antimicrobials and the conditions for optimum inhibitory effects against *C. perfringens* spores and vegetative cells.

EOs. Essential oils (EOs) are secondary metabolites of aromatic plants having the characteristics of being volatile, natural, complex compounds with strong odors (56, 57). Historically, EOs have been used for thousands of years as food preservatives, for medicinal purposes, and for flavor and aroma. Many EOs have been shown to have antimicrobial activities against a broad range of bacteria, including *C. perfringens* (58). Cinnamaldehyde is one of the most common plant-derived EOs; it is listed as GRAS by the Flavor and Extract Manufacturers Association (FEMA) (59) and exhibited strong antibacterial activity against *C. perfringens* vegetative cells (Tables 1 and 2) (60). Cinnamaldehyde (≥0.5%, wt/vol) was also effective in controlling *C. perfringens* spore germination and outgrowth in cooked ground beef and ground turkey during abusive chilling conditions (61, 62).

Carvacrol, thymol, and oregano oils are the key components in the EOs of oregano and thyme (58). The inhibitory effect of these EOs against germination and outgrowth of *C. perfringens* spores has been evaluated after deliberately contaminating cooked ground meats with *C. perfringens* spores prior to cooling in a deviated process from the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) stabilization guidelines for cooling meat products (61, 62). Carvacrol (1 to 2%, wt/vol), thymol (1 to 2%, wt/wt), and oregano oil (2%, wt/wt) effectively restricted *C. perfringens* growth from spores during 12 to 21 h of cooling from 54.4 to 7.2°C, with less than 1 log

CFU/g increase in the bacterial count in cooked ground beef (61). Another study, by Juneja and Friedman, showed that higher concentrations (2%, wt/wt) of carvacrol, thymol, and oregano oils were needed to completely restrict the growth of *C. perfringens* spores following more than 15 h of cooling of cooked ground turkey (62). This clearly indicates that the inhibitory effects of these EOs are variable and depend on the type and source of meat. Also, both thymol and carvacrol showed strong antibacterial activity against *C. perfringens* cells under *in vitro* conditions (Table 2) (63). Although *in vivo* studies with a chicken model showed that the supplementation of food with thymol and carvacrol did not reduce the number of *C. perfringens* in the chicken, it caused the reduction of the numbers of other bacteria and intestinal lesions in the gut and also enhanced the intestinal integrity and immune response (63, 64).

An extensive study by Si et al. with 66 different EOs demonstrated that 33 EOs inhibited >80% *C. perfringens* growth, while 9 EOs showed 50 to 80% inhibition and 24 EOs showed <50% inhibition (65). Out of the 33 EOs that exhibited >80% inhibition, seven were further investigated in a chicken model for their inhibitory potency against *C. perfringens* under acidic conditions. Excluding citral oil and geraniol, the inhibitory activities of the other five compounds (2-*tert*-butyl-6-methylphenol, carvacrol, chamomile roman oil, citronellal, and *trans*-cinnamaldehyde) were stable under acidic conditions (pH 2.0) (65). A few other studies also reported antimicrobial activity of some of the EOs against the *C. perfringens* growth (66–68). All these studies collectively suggest the potential use of EOs to reduce the number of *C. perfringens* organisms in food products.

Tannins. Tannins are water-soluble phenolic compounds that can be found in a variety of plants and are used as food additives or in leather processing industries due to their profound antimicrobial activity (69, 70). The effects of tannic acid and its hydrolytic product, gallic acid, were evaluated against vegetative cells of *C. perfringens* strain ATCC 13124 (71). The results showed that, tannic acid exhibited significant inhibition (lowest inhibitory concentration, 3.1 μM) of *C. perfringens* growth at concentrations greater than 10 μg/ml, while gallic acid showed no observable growth inhibition at concentrations up to 1,000 μg/ml (71). This result suggests that the chemical structure of tannic acid is vital for retention of its antimicrobial activity. The source of tannins may also have an impact on their antimicrobial activity against *C. perfringens*. One study reported that the tannins from chestnut had higher antibacterial activity against *C. perfringens* than tannins from quebracho plants (72). However, tannins from both chestnut and quebracho could reduce the cytotoxicity of two *C. perfringens* toxins, alpha toxin and epsilon toxin, in MDCK cells (72). *C. perfringens* also showed no or very minimal resistance development against tannins over time compared to other antimicrobial products (73).

Green tea extracts. Green tea is one of the most consumed beverages worldwide and has beneficial effects on human health. It shows some antimicrobial activity against a wide range of bacteria, including *C. perfringens* (74). The activities of different polyphenols from extracts of green tea leaves against the growth of *C. perfringens* ATCC 13124 were evaluated by the disc diffusion method, and it was found that gallocatechin and epigallocatechin showed the strongest growth inhibitory activity (75). The inhibitory effect of green tea extracts against germination and outgrowth of *C. perfringens* spores in different cooked meat products during deviated chilling regimens up to 21 h was also investigated (76). At 2.0%, the green tea extract with higher catechin content was successful in arresting growth from spores in all tested meats during a 21-h cooling period from 54.4 to 7.2°C (76). However, the efficacy of green tea extracts to prevent *C. perfringens* spore germination and outgrowth seems to be concentration and condition dependent (a higher dose of green tea extracts prevents *C. perfringens* growth in meat products for a longer storage time) (76). The catechin content of extracts is considered the key factor for the observed antibacterial activity, and this may partly explain the lack of activity of green tea extract with a lower total catechin content.

Fatty acids. Antimicrobial activity of short- and long-chain fatty acids against *C. perfringens* growth has been demonstrated in a few studies (77–79). However, long-chain fatty acids have been found to be more effective than short-chain fatty acids in inhibiting the growth of *C. perfringens* (77, 78). Saturated lauric acid ($C_{12:0}$) and unsaturated linoleic acid ($C_{18:2}$) at 0.5 mmol/liter were able to completely inhibit *C. perfringens* (77). A more recent study by Skøivanová et al. determined the susceptibility of vegetative cells of *C. perfringens* ATCC 13124 and CNCTC5459 (a clinical isolate) to different short- to long-chain fatty acids (79). Among them, caprylic ($C_{8:0}$), capric ($C_{10:0}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$), and oleic ($C_{18:0}$) acids could effectively inhibit growth of both strains, and linoleic acid ($C_{18:2}$) inhibited only strain CNCTC5459 and not strain ATCC 13124 (79). Lauric acid ($C_{12:0}$) was proven to be the most potent antimicrobial, followed by myristic acid ($C_{14:0}$) and capric acid ($C_{10:0}$) (79). In an *in vivo* study, Timbermont et al. showed that the incidence of necrotic enteritis caused by *C. perfringens* in broiler chicken decreased if chickens were fed a diet supplemented with butyric acid in combination with lauric acid, thymol, cinnamaldehyde, and EO of eucalyptus at a lower dose (80).

Lysozyme. Lysozyme is a bacteriolytic enzyme that is obtained mainly from hen egg white and represents 3 to 5% of the egg albumin protein. It received GRAS status as a direct food additive and has been proven to be nontoxic to humans (36, 55). Lysozyme possesses a broad-spectrum antimicrobial activity and is commonly used in cheese production to prevent the late-blowing type of defect caused by *Clostridium* spp. (81, 82). Lysozyme inactivates bacterial cells by weakening the cell wall peptidoglycan (PG) layers via the hydrolysis of the β-1,4 linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid, thus making it an effective antimicrobial agent against numerous Gram-positive bacteria (54, 83, 84). Lysozyme can inhibit the growth of *C. perfringens* vegetative cells at 156 μg/ml (85) (Table 1). However, sublethal concentrations of lysozyme could inhibit the production of toxins (85). Based on the study of the effect of lysozyme on *Alicyclobacillus acidoterrestris*, it was hypothesized that lysozyme may inactivate spores by causing a rapid hydrolysis of the spore cortex, resulting in damage of the spore core (86). This notion may be supported by the fact that lysozyme can degrade the cortex of decoated *C. perfringens* spores lacking the cortex lytic enzyme SleC, leading to the resumption of wild-type-level colony-forming efficiency of *sleC* spores (87).

Chitosan. Chitosan is a biopolymer derived from the partial de-N-acetylation of chitin, which is the key component in the exoskeletons of crustaceans and is regarded as the second most abundant biopolymer in nature, following cellulose (88). It is nontoxic, nonantigenic, biocompatible, and biofunctional, and it has been approved as a food additive for many years. Moreover, its antimicrobial property makes it a potential food preservative in a variety of food products (89). A study by Juneja et al. demonstrated that 3% chitosan (degree of acetylation, 0.14) can reduce germination and outgrowth of spores of *C. perfringens* in cooked beef and turkeys by 4 to 5 log units during extended chilling regimens up to 18 h (90).

Nisin. Nisin, a class I bacteriocin and a polypeptide of 34 amino acid residues produced by certain strains of *Lactococcus lactis* subsp. *lactis*, has been successfully used as a food preservative in a variety of food products (91). It is approved by the FDA and WHO as GRAS and is permitted to be used in various food products in more than 50 countries. Nisin is an attractive option as a food additive because of its natural source and high effectiveness against a broad spectrum of Gram-positive bacteria. Several reports demonstrated the inhibitory activity of nisin alone or in conjunction with other food preservation technologies, such as heat and HHP, in controlling spores of *Clostridium* spp. in a variety of food products (26, 92). Nisin exerts its inhibitory effect against spore outgrowth and vegetative growth of *C. perfringens* FP and NFB isolates under laboratory conditions, but in a meat model system no such effect was observed (92). Nisin causes the lengthening of the lag phase of vegetative growth of *C. perfringens* type A strain NCTC8798 in a concentration-dependent manner (92). The higher the

concentration of nisin, the longer the lag phase observed. Nisin at 500 IU/ml or higher is thus required to prevent growth of *C. perfringens* NCTC8798 for up to 2 weeks (92).

Lacticin. Another type of class I bacteriocin is the two-component heat-stable lantibiotic lacticin 3147, produced by strains of *Lactococcus lactis* DPC 3147. Lacticin 3147 was reported to have a broad-spectrum inhibition toward Gram-positive bacteria, similar to that of nisin (93), but it is distinct from nisin in terms of its solubility and being active at physiological pH (94). It has been reported that the combination of lacticin and 2% sodium lactate was effective in reducing *C. perfringens* growth in fresh pork sausage (95).

Pediocin. Pediocins are well-known class II-type bacteriocins that are produced by *Pediococcus* spp. These are small, heat-stable, non-lanthionine-containing membrane active peptides (96). Pediocins are also affirmed as GRAS additives for application in certain food products (55). Different types of pediocins showed inhibitory activities against *C. perfringens* growth, such as pediocin AcH and pediocin PA-1 from *Pediococcus acidilactici* and pediocin A from *Pediococcus pentosaceus* (96–98). Pediocin PA-1 could lower the counts of *C. perfringens* by 2 and 0.8 log units, respectively, when frankfurters were stored at 10°C for 60 days and at 15°C for 30 days (97). Grilli et al. showed that a diet supplemented with pediocin A significantly improved the growth of broilers challenged with *C. perfringens* for up to 42 days (98).

GERMINATION-INDUCED INACTIVATION STRATEGIES

Having intrinsic resistance properties, *C. perfringens* spores can survive in the environment for extended periods (i.e., decades), and once these dormant spores come in contact with small molecules termed germinates, they return to being vegetative cells via the germination process (99, 100). In recent years, significant progress has been made in understanding the mechanism of *C. perfringens* spore germination, leading to the identification and characterization of suitable germinants and germinant receptors for spores of *C. perfringens* FP and NFB strains (99, 101). Although there are differences in the preference of germinants among the strains, some germinants (such as AK or L-cysteine) can induce germination of spores of a wide range of *C. perfringens* strains (99, 102). Such understanding has led to the development of novel strategies involving induction of spore germination followed by subsequent killing of germinated spores with mild treatments (39, 92, 103, 104), as in the following examples. (i) The application of germinant AK in meat products followed by HHP treatment (586 MPa) at high temperature (73°C for 10 min) significantly killed *C. perfringens* spores in the meat products (103) (Table 1). (ii) Permissive levels of chemical preservatives (such as nisin, sorbate, and benzoate) effectively arrested outgrowth of germinated *C. perfringens* spores in rich medium. However, higher levels of chemicals were needed to achieve significant inhibitory effects against *C. perfringens* spores inoculated into chicken meat (39, 91). (iii)) Triggering spore germination considerably increased the sporicidal activity of commonly used disinfectants against *C. perfringens* FP spores attached to stainless steel chips (99). In addition, this germination-induced inactivation strategy also proved to be effective in killing spores from other *Clostridium* species (105–107). Collectively, triggering spore germination followed by inactivation treatment represents a novel strategy to enhance killing of *Clostridium* spores.

CHALLENGES OF INACTIVATION STRATEGIES AND FUTURE DIRECTIONS

Although there has been significant success in inactivating *C. perfringens* vegetative cells, the inactivation or elimination of *C. perfringens* spores from food products still remains a challenge. This is mostly because the effect of extreme physical stress damages the food quality and the use of excessive chemical preservatives results in toxicity and causes harm to human health. Furthermore, the addition of antimicrobial agents into food products might exhibit nonspecific inhibitory activity toward beneficial microorganisms harbored in the GI tract, causing an imbalance in the gut microflora (108, 109). These issues emphasize the importance of identifying the active compounds

and evaluating their toxicity prior to application to food products in order to warrant their safe usage. In addition, the elucidation of mechanisms of action of different physical and chemical techniques and naturally derived antimicrobials is critical for the efficient utilization of these treatments against the growth of *C. perfringens* spores and vegetative cells.

The recently developed germination-induced inactivation strategies also have some challenges (26, 103, 104). For example, due to spore germination heterogeneity, in a spore population some spores germinate within minutes and some takes hours or even longer to germinate (110). Therefore, further studies are needed to improve germination conditions for superdormant spores that exhibit slow or no germination and to validate the effectiveness of the germination-induced inactivation strategy under the practical conditions of food industry environments.

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